Intracellular signaling of transcription and secretion of type IV collagen after angiotensin II-induced cellular hypertrophy in cultured proximal tubular cells

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Physiologic concentrations of angiotensin II (All) can induce cellular hypertrophy in murine proximal tubular epithelium (MCT cells). This response is characterized by an increase in cell size, new protein synthesis, and by the secretion of new basement membrane type IV collagen in the absence of cellular proliferation. The present study was undertaken to evaluate the second messengers of these All-induced cellular events with special reference to the increase in type IV collagen secretion. In initial experiments we observed that pretreatment of MCT cells with agents that increase concentrations of intracellular cAMP, like forskolin, dibutyryl cAMP, and isobutyl-methyl-xanthine abolish All-induced amino acid incorporation, but have no effect on control cells or on their proliferation. In addition, 10^{-8} M All significantly decreased the concentration of intracellular cAMP. Phorbol esters were without significant effect on the hypertrophy or proliferation of All-stimulated MCT cells or their rested controls. The transfection of MCT cells with reporter genes containing regulatory elements for type IV collagen revealed that the stimulatory effects of All on collagen type IV depend, at least to some extent, on an increase in gene transcription. Agents increasing intracellular cAMP concentrations inhibited the All-induced increase in transcription and secretion of collagen type IV, but had no effect on MCT cells grown in media without All. Our findings provide evidence that All-induced changes in tubular epithelium leading to the secretion of type IV collagen are mediated by a decrease in intracellular cAMP.

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Introduction

Renal proximal tubular cells in adult kidneys hypertrophy after a reduction in nephron mass (Wolf and Neilson, 1991). Such epithelium rests ordinarily on a complex basement membrane composed of a variety of constituents, including substantial amounts of type IV collagen. The establishment of renal proximal tubular cells in serum-free cultures (Haverty *et al.*, 1988) has greatly facilitated the study of cytokines on their paracrine cellular processes. We have recently reported that angiotensin II (All) can induce cellular hypertrophy, but not proliferation in murine proximal tubular epithelium (MCT) cells, a cultured proximal tubular cell line (Wolf and Neilson, 1990). Hypertrophy can be collectively defined by determining cell size, total protein content, and [3 H]leucine incorporation into newly synthesized proteins and by the secretion of specific proteins like basement membrane-associated type IV collagen. Such All-induced events follow the binding of specific All receptors on MCT cells and do not involve an increase in eicosanoid synthesis. Associated increases in the synthesis of type IV collagen presumably supports the cytoskeletal remodelling associated with a change in cell size.

The second messenger systems involved in All-mediated effects in proximal tubular cells have been investigated principally in studies analyzing changes in solute transport (Liu and Cogan, 1987, 1989). Recent work, e.g., suggests that All-mediated changes in ion transport in the proximal tubule are facilitated, in part, by a decrease in intracellular cAMP (Woodcock and Johnson, 1982; Liu and Cogan, 1989). It is proposed that All receptors on renal tubular cells interface with inhibitory G-proteins (G_i) and adenylate cyclase (Liu and Cogan, 1989). In this setting, the binding of All to its receptor would stimulate G_i -proteins, resulting in a down-regulation of adenylate cyclase. In contrast, in other renal cells such as mesangial cells, All transduced effects involve activation of phospholipase C and the phosphatidylinositol messenger systems (Douglas 1987, Sekar *et al.*, 1990).

Figure 1. Effect of All on cellular size and DNA content. Treatment of rested cells for 48 h with 10^{-8} M All. DNA was stained with propidium iodide after RNase treatment. Cells ($\times 10^4$) were analyzed in rested control cells and cells treated with All. Resulting scans were expressed as contour plots after computer analysis. The shift of the plot on the y axis indicates an increase in cellular size, but not of DNA content after All treatment.

The present study was undertaken to evaluate some of the early signal transduction events supporting the secretion of type IV collagen during All-induced protein synthesis leading to the hypertrophy of proximal tubular cells.

Results

Effects of phorbol ester and cAMP modifying agents on All-induced protein synthesis and proliferation

Because All can transmit signals in several types of cells through the phosphatidylinositol/protein kinase C pathway or by lowering intracellular cAMP concentrations, we were interested in whether agents interacting with these pathways would interfere with All-induced cellular events. First, we confirmed, in a dose capable of producing hypertrophy (Figure 1), that the incubation of rested MCT cells with 10^{-8} M All for 60 min reduced significantly intracellular levels of cAMP as detected by radioimmunoassay (Figure 2). In contrast, PTH treatment in the same experiment enhanced significantly intracellular cAMP content, suggesting that adenylate cyclase in MCT cells is under a bidirectional regulation found normally in proximal tubules *in vivo* (Milanes *et al.*, 1989).

Since cellular hypertrophy, at its maximal

point, normally only increases cell volume by 15–20% (Wolf and Neilson, 1990), we have found that routine measurements of changes in cell size by fluorescent-activated cell sorting (FACS) are not sufficiently sensitive or quantitative to be useable as an end-point assay. The process of hypertrophy, however, is also characterized by an increase in new protein synthesis without an increase in the content of cellular

Figure 2. Intracellular cAMP concentrations in MCT cells. Treatment of rested cells with 10^{-8} M All significantly reduced intracellular cAMP concentrations as measured in cellular lysates with a specific radioimmunoassay. In contrast, PTH treatment increased intracellular cAMP concentrations in MCT cells. * $p < 0.05$ vs untreated controls, $n = 5$.

DNA, and [^3H]leucine incorporation into proteins synthesized de novo is closely linked to changes in total cellular protein and cell size in MCT cells (Wolf and Neilson, 1990).

Although pretreatment of smooth muscle cells in culture with 200 nM phorbol-12,13-dibutyrate (PDB) for 12 h can down-regulate protein kinase C activity by $\sim 90\%$ (Taubman *et al.*, 1989), the same dose does not significantly alter [^3H]leucine incorporation in controls or All-stimulated cells (Figure 3A). Similar treatments with phorbol-12-myristate-13-acetate (PMA) also did not alter the All-induced increases in protein synthesis in MCT cells. Phorbol esters were, in fact, without significant effect on proliferation in control or All-treated cells (Figure 3B).

All reduces intracellular cAMP normally after receptor binding in membrane preparations isolated from renal proximal tubule cells (Douglas, 1987). Pertussis toxin-sensitive inhibitory G_i -proteins are also involved in the transduction pathways which down-regulate adenylate cyclase (Liu and Cogan, 1987). We found in our previous investigation that the addition of pertussis toxin to cultures blunted the All-induced hypertrophy in MCT cells (Wolf and Neilson, 1990). To investigate these issues further, 100 and 500 μM of the relatively stable cAMP analogue dibutyryl-cAMP (db-cAMP) totally abolished the All-stimulated increase in [^3H]leucine incorporation, but did not alter protein synthesis in rested, control cells. In addition, the adenylate cyclase activator forskolin (FK), and the phosphodiesterase inhibitor isobutyl-methyl-xanthine (IBMX) both attenuated All-stimulated [^3H]leucine incorporation. Higher concentrations of IBMX (500 μM), however, inhibited [^3H]leucine incorporation in rested, control cells. db-cAMP, IBMX, and FK were without effect on cellular proliferation (Figure 3B).

Effect of phorbol ester and cAMP-modifying agents on the secretion and transcription of type IV collagen

We have shown previously that, as part of hypertrophic events in MCT cells, All can stimulate the secretion of basement membrane type IV collagen into culture supernatants without influencing the secretion of type I collagen (Wolf and Neilson, 1990). Agents increasing intracellular cAMP, but not phorbol ester, abolished significantly the All-induced secretion of collagen IV in MCT cells (Table 1). As shown in Figure 4, 5–50 ng/ml of pertussis toxin also abolished,

in a dose-dependent fashion, the All-induced secretion of type IV collagen. Pertussis toxin (50 ng/ml) had no effect on control cells (210 ± 80 ng/ml collagen type IV, $n = 10$).

Reporter gene constructs containing enhancer-promoter elements from murine collagen type IV linked to the CAT-gene were transiently transfected into MCT cells using DEAE-dextran. Figure 5 shows a representative autoradiogram of a thin-layer chromatography from one of these chloramphenicol acetylation assays. Incubation of 10^{-8} M All for 24 h induced an increase in CAT activity when compared with cells grown in medium without All. This increase likely reflects changes in gene transcription for type IV collagen. The All stimulation of transcription seems to be receptor mediated, since pre- and co-treatment of cells with the specific All-receptor blocker saralasin partly attenuated changes in transcription (Table 2). In addition, the small increase in CAT activity after incubation with saralasin alone is in agreement with previous observations that saralasin seems to have some partial agonist activity. db-cAMP, IBMX, and FK also blocked the All-induced increase in CAT activity, but had no effect on control cells.

Discussion

In the kidney, the effects of All have been investigated principally within the context of hemodynamic events or for its influence on ion transport (Liu and Cogan 1987, 1989). Two different kinds of renal All-receptors coupled to dissimilar second-messenger systems have been described in such experiments. All-receptors in mesangial cells, e.g., stimulate phospholipase C leading to subsequent signaling through the phosphatidylinositol pathway (Sekar *et al.*, 1990). In tubular cells, however, All-receptors inhibit adenylate cyclase (Douglas, 1987). The present study provides new evidence that events occurring during All-induced cellular hypertrophy in MCT cells are mediated, in part, by a decrease in intracellular levels of cAMP. Agents that raise intracellular cAMP concentrations also abolish the All-induced increase in protein synthesis independent of cellular proliferation. Moreover, intracellular cAMP concentrations decreased significantly after All stimulation in MCT cells. The measured concentrations of intracellular cAMP in murine MCT cells after All stimulation are in good agreement with values recently reported from cultured rabbit proximal tubular cells (Friedlander *et al.*,

A.

B.

Figure 3. Effect of intracellular cAMP modifying drugs on [³H]leucine incorporation into proteins (A) and on [³H]thymidine into DNA (B) of MCT cells stimulated with 10^{-8} M AII for 48 h or rested controls. Significant inhibition of the AII stimulated [³H]leucine incorporation into proteins by agents increasing intracellular cAMP concentrations (A). However, dibutyl cAMP (db-cAMP), iso-butyl-methyl-xanthine (IBMX), and forskolin (FK) had no significant effect on proliferation of MCT cells (B). Phorbol ester treatment (PMA or PDB) was without effect on [³H]leucine or thymidine incorporation in AII stimulated cells or controls. All concentrations are in micromolars except for PMA and PDB which are in nanomolars. * $p < 0.05$ vs untreated controls, * $p < 0.05$ vs MCT cells treated with AII only, † $p < 0.01$ vs MCT cells treated with AII only; $n = 8$ for [³H]leucine and $n = 12$ for [³H]thymidine incorporation experiments.

1990). On the other hand, intracellular cAMP significantly increased after parathyroid hormone (PTH) treatment, a ligand known to stimulate adenylate cyclase in proximal tubular cells (Milanes *et al.*, 1989).

We found with FACS analysis (Wolf and Neilson, 1990 and Figure 1) that AII increased the cell size by ~20–30%. Pretreatment with db-cAMP blocked this increase by one-third (data not shown). Thus, FACS analysis is not really sensitive enough to detect small, incremental changes in cell volume, and we, therefore, resorted to using de novo leucine incorporation as a marker of protein synthesis (Wolf and Neilson, 1990).

It has been proposed that the AII-receptor in proximal tubular cells is coupled to adenylate cyclase through a pertussis toxin-sensitive G_i protein (Liu and Cogan, 1989). Previous data indicated that AII-induced hypertrophy was

abolished by pertussis toxin, underscoring the importance of a decrease in intracellular cAMP in this model of hypertrophy (Wolf and Neilson, 1990). In an in vivo model of compensatory renal

Table 1. Collagen type IV secretion

Agents	Controls (ng/ml)	10^{-8} M AII (ng/ml)
0	264 ± 135	778 ± 360*
100 μ M db-cAMP	120 ± 41	176 ± 60†
500 μ M db-cAMP	99 ± 69	138 ± 96†
100 μ M IBMX	94 ± 31	141 ± 41†
500 μ M IBMX	52 ± 34*	72 ± 47†
200 nM PMA	310 ± 77	706 ± 280
200 nM PDB	185 ± 47	430 ± 114

* $p < 0.05$ vs untreated controls; † $p < 0.01$ vs cells treated with AII only ($n = 10$).

Figure 4. Dose-dependent inhibition of All stimulated collagen type IV secretion by pertussis toxin. Control cells were incubated in medium without All. Pertussis toxin (50 ng/ml) had no effect on control cells. MCT cells were stimulated with 10^{-8} M All in the presence of a single dose of 0–50 ng/ml pertussis toxin for 24 h. (●), rested control cells, (○), cells treated with 10^{-8} M All in the presence of various concentrations of pertussis toxin. * $p < 0.01$ vs control cells, * $p < 0.01$ vs All-stimulated cells without pertussis, ** $p < 0.005$ vs All stimulated cells without pertussis, $n = 10$.

hypertrophy after uninephrectomy in rats, PTH-dependent adenylate cyclase activity in cortical membranes isolated from kidneys with compensatory renal growth was significantly lower compared with controls (Milanes *et al.*, 1989). These findings suggest that a decrease in intracellular cAMP might play a role in compensatory renal hypertrophy in vivo. A similar decrease in cAMP accompanied by a rise in cGMP was described in the contralateral hypertrophic kidney after uninephrectomy in an earlier study (Schlondorff and Weber, 1978), although other authors could not confirm these data (Bergeron and Hoang, 1983). Liu and Cogan (1990) provided further evidence for dual All signals in microperfused rat proximal tubules. In this system, one-third of the All-induced bicarbonate absorption was due to signaling through protein kinase C, whereas the remaining two-thirds was mediated through a decrease in cAMP (Liu and Cogan, 1990). In contrast, other investigators found no evidence that All activates phosphoinositol breakdown in intact rat proximal tubules (Sekar *et al.*, 1990). Beside obvious species differences, it is rightly difficult to compare in vivo studies investigating All effects on fluid absorption in intact animals with All actions in cultured proximal tubular cells.

We have shown in our previous study (Wolf and Neilson, 1990) that 10^{-8} – 10^{-10} M All can induce hypertrophy in MCT cells. Although in vivo systemic concentrations of All are in the

picomolar range (Cogan, 1990), there is recent evidence that intrarenal concentrations of All measured in proximal tubules and star vessels of rats by micropuncture, are 34–41 (proximal tubules) and 95 (star vessels) nM, respectively (Seikaly *et al.*, 1990). These intrarenal values are very close to the 10^{-8} M All used in the present study, which was the optimum dose for inducing hypertrophy in cultured murine proximal tubule cells (Wolf and Neilson, 1990). Furthermore, MCT cells have specific All receptors with a dissociation constant in the nanomole range (Wolf and Neilson, 1990), close to the All con-

Figure 5. Representative autoradiogram of a thin-layer chromatography plate after separation of acetylated [14 C]chloramphenicol (CAT activity) from unacetylated forms. The CAT assay demonstrates the transcriptional effect of 10^{-5} – 10^{-10} All on MCT cells transfected with plasmid pR184 containing $\alpha 1$ (IV) enhancer/promoter elements. As a positive control, cells were transfected with pSV₂CAT containing the CAT gene linked to the SV-40 promoter and enhancer. pA₀CAT containing only the CAT gene served as a negative control.

Table 2. Quantification of CAT assay

Reporter gene constructs	Relative CAT activity
pR184 + control medium	1.14
pR184 + 10^{-5} M All	1.90
pR184 + 10^{-6} M All	3.10
pR184 + 10^{-10} M All	1.10
pR184 + 10^{-6} M saralasin	2.80
pR184 + 10^{-6} M saralasin + 10^{-8} M All	1.80
pR184 + 200 μ M db-cAMP	1.40
pR184 + 200 μ M db-cAMP + 10^{-8} M All	1.10
pR184 + 200 μ M IBMX	1.10
pR184 + 200 IBMX + 10^{-8} M All	1.02
pR184 + 40 μ M FK	0.90
pR184 + 40 μ M FK + 10^{-8} M All	1.01

Mean values of three independent experiments. pA_0 , the negative control was assigned a relative value of 1.00; pSV_2 , the positive control, was assigned a value of 7.41. SEMs were <5%.

centration used in this study. Why 10^{-5} M All had less effect on type IV collagen transcription than 10^{-8} M, in the present study, is not entirely clear, but some suggestions can be made. Higher doses of All, over a prolonged period of time, might downregulate the All receptor (Douglas, 1987). A previous study using cultures of rabbit proximal tubular cells has also demonstrated that higher concentrations of All (10^{-5} – 10^{-6} M), but not 10^{-8} M led to a rise in cytosolic Ca^{2+} (Norman *et al.*, 1987). One could speculate that a similar increase in intracellular Ca^{2+} in MCT cells, in the presence of higher concentrations of All, might partially block or counteract the stimulatory effects of a decrease in cAMP on cellular hypertrophy and on the transcription of type IV collagen.

Although we did not directly measure the products of the phosphatidylinositol pathway, like inositol triphosphate and diacylglycerol, phorbolesters used in a concentration found by other investigators to interact with protein kinase C neither modulated All-induced hypertrophy, nor had significant effect in control cells in our system. Since we performed only down-regulation experiments of protein kinase C, we can not formally exclude a role of the phosphatidylinositol pathway in our system. The general notion, however, that All *in vivo* is not a major stimulant for the phosphatidylinositol signal transduction system in proximal tubular cells and our observed effects with cAMP modifying agents suggest that protein kinase C does not have a major role in the All-induced hypertrophy in MCT cells.

All also stimulates the biosynthesis of collagen IV, a basement membrane-specific protein, but not procollagen I in cultured MCT cells (Wolf and Neilson, 1990). We believe that an increase in collagen IV transcription and biosynthesis is an appropriate consequence of cellular hypertrophy, because hypertrophic cells probably enlarge the basal surface adherent to the basement membrane. Increased basement membrane synthesis may be necessary to accommodate the increased cellular mass. The stimulatory effect of All on collagen IV was abolished by treatment with exogenous db-cAMP, the phosphodiesterase inhibitor IBMX and FK, but not through PMA and PDB. Moreover, agents increasing intracellular cAMP and phorbolster did not significantly influence collagen IV secretion in rested control cells. Pertussis toxin inhibited, in a dose-dependent fashion, the All-induced stimulation of collagen IV secretion. These findings underscore the notion that All-induced collagen secretion is mediated by a decrease in intracellular concentrations of cAMP, because pertussis toxin interrupts the inhibition of adenylate cyclase after All-receptor binding. Transfection of MCT cells with reporter gene constructs, in which enhancer/promoter elements from the $\alpha 1(IV)$ gene are linked to the CAT gene, revealed that the stimulatory effect of All on type IV secretion is, at least to some extent, due to an increase in transcriptional activity. Furthermore, agents increasing intracellular cAMP concentrations seem to block the All-induced transcription of type IV, but had no effect on cells treated with control medium only. The effects of cAMP on collagen IV synthesis is highly cell type-dependent and some controversies exist in the literature. For example, db-cAMP depresses collagen synthesis in lung and foreskin fibroblasts, whereas the same substance has stimulatory effects on type IV collagen synthesis in PFHR-9 cells (Bush and Benson, 1987). cAMP and retinoic acid also stimulate the expression and synthesis of type IV collagen in cultured murine teratocarcinoma cells (Strickland *et al.*, 1980). cAMP alone, however, has no stimulatory effect on collagen IV synthesis in F9 cells.

We are aware of the intrinsic problems linking early signal transduction events which take seconds to minutes with the observed effects of All on protein synthesis and collagen secretion 24–48 h later. However, the down-regulation of adenylate cyclase may require an extended interval to see the subsequent hypertrophy. The mechanism by which cAMP me-

diates cellular hypertrophy in MCT cells is unclear.

One could argue that agents increasing cAMP might interfere with sodium-coupled amino acid uptake in MCT cells and therefore inhibit protein synthesis. Agents, however, that increase cAMP, like IBMX and FK, had no effect on protein synthesis in unstimulated MCT cells or on proliferation in unstimulated or All-stimulated cells. These agents, furthermore, blocked All-induced transcription and secretion of collagen IV. Such findings support a previous notion that the effects of cAMP can not be explained simply by a nonselective inhibition of ion-coupled transport systems (Saccomani *et al.*, 1990). Such membrane-protein systems, however, can be kept inactivated by phosphorylation from cAMP-dependent kinase (Weinman *et al.*, 1989). Furthermore, as a hypothesis, nuclear proteins such as regulatory trans-acting factors, may also be phosphorylated and attenuated by similar enzyme events (Taylor, 1989). Lowering intracellular cAMP concentrations after All stimulation could, therefore, dephosphorylate these trans-acting proteins and induce a genetic program leading to cellular hypertrophy. We are currently investigating potential genes expressed in All stimulated hypertrophy. The isolation of such genes would then allow better insight into the regulatory mechanisms mediated by cAMP in All-induced hypertrophy.

Materials and methods

Cell culture

The characterization of MCT cells through microdissection and immunoaffinity methods has been described in detail elsewhere (Haverty *et al.*, 1988, 1989). MCT cells were carried in culture as a differentiated cell line in Dulbecco's modified Eagle's medium (DMEM) with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY). Cells were passaged every 48–72 h. For individual experiments, MCT cells were rested for 48 h in a 50:50 mixture of DMEM/Ham's F12 medium without serum.

Measurement of DNA synthesis

[³H]thymidine incorporation into DNA was used as a measurement of DNA synthesis. Previous studies have demonstrated that this method closely reflects the proliferative response in MCT cells (Haverty *et al.*, 1988). MCT cells ($\times 10^4$) were rested for 48 h in serum-free media and then stimulated with daily doses of 10^{-8} M All or control medium in the presence or absence of a single dose of the following factors for another 48 h: 100–500 µM N,2-O-dibutyryl adenosine 3'-5'-cyclic monophosphate (db-cAMP), 100–500 µM 3-isobutyl-1-methyl-xanthine (IBMX), and 10–50 µM forskolin (FK, all substances from Sigma, St. Louis, MO). Cells were incubated for the last 6 h with 1 µCi [³H]thymidine/well (5 Ci/mmol; Amersham, Arlington Heights, IL). In addition, quiescent MCT cells were pretreated with 200 nM PDB or with

200 nM PMA (Sigma) for 12 h to deplete cells of protein kinase C. Cells were then subsequently stimulated with 10^{-8} M All in the presence of a new phorbol ester dose for 48 h including [³H]thymidine for the last 6 h. Cells were harvested with an automatic cell harvester (Brandel, Gaithersburg, MD) on glassfiber filters. Filters were counted in a LKB 1209 Rackbeta counter (Pharmacia-LKB, Piscataway, NJ) in scintillation cocktail. Data are reported as counts per well.

[³H]leucine incorporation into newly synthesized proteins

Incorporation of [³H]leucine into trichloroacetic acid (TCA)-precipitable proteins was considered as an assessment of de novo protein synthesis, accurately reflecting changes in total protein content determined by other methods (Wolf and Neilson, 1990). MCT cells (5×10^5) were transferred into 24-well plates and rested in serum-free media for 48 h. After stimulation with daily doses of 10^{-8} M All for 48 h, in the presence or absence of a single dose of the above described factors, cells were pulsed for the last 12 h with 5 µCi [³H]leucine (142 Ci/mmol, Amersham). Similar to DNA synthesis experiments, cells were pretreated with phorbol ester and subsequently stimulated with All. After pulsing, MCT cells were washed three times in ice-cold phosphate-buffered saline (PBS), precipitated twice in 10% TCA, redissolved in 0.5 M NaOH with 0.12% Triton X-100 and counted in 10 ml Aquasol-2 scintillation cocktail (NEN-Research Products, Boston, MA).

Cell cycle analysis by cytofluorography (FACS)

For determination of cell size and DNA content, cultured MCT cells were washed three times in ice-cold PBS, fixed in 1% paraformaldehyde (Baker Chemicals, Phillipsburg, NJ), permeabilized for 3 min in 1% Triton-X 100 (Sigma) and treated for 30 min at 37°C with 150 U RNase I-AS (Sigma) to digest all cellular RNA. Remaining cellular DNA was then stained with propidium iodide (Sigma, 50 µg/ml PBS). After washing with PBS, 10 000 cells were analyzed using a FAC-Scan (Becton-Dickinson, Mountain View, CA) with FSC: E00/1.00, SCC: 270/1.00, and FL2: 420/1.00. Forward cell scatter (FSC) was considered as a measurement of cell size. Data were analyzed with Hewlett-Packard software and represented as contour plots.

Radioimmunoassay of collagen type IV

Collagen type IV in the cell culture supernatants of MCT cells was measured by radioimmunoassay using a monospecific polyclonal anticollagen IV antibody as previously described (Haverty *et al.*, 1988). Cells were treated either with control medium or 10^{-8} All for 24 h in the presence or absence of 100/500 µM db-cAMP, 100/500 µM IBMX, 200 nM PMA, 200 nM PDB, or 5–50 ng/ml pertussis toxin (Sigma). Data were expressed as nanograms per milliliter, normalized for cell number.

Intracellular cAMP concentrations

Rested MCT cells (2×10^5) were treated for 60 min with either control medium, 10^{-8} M All or 10^{-8} M PTH (Sigma). After harvesting, cells were washed, counted, lysed, and deproteinized in 100% ethanol with 0.1 M HCl. Cyclic AMP was measured in duplicates from each experiment ($n = 5$ independent stimulation experiments) in the supernatant with a commercial competitive radioimmunoassay (Amersham). Unbound cAMP was separated from bound material

by charcoal treatment. Data are expressed as picomoles cAMP/10⁶ cells.

Transfections of cells and chloramphenicol acetyl transferase (CAT) reporter gene assay

The plasmids used for transfections were as follows: pA₀CAT containing only the CAT gene as a negative control; the pSV₂CAT containing the CAT-gene linked to the SV-40 promoter and enhancer was used as a positive control for transfection efficiency; and pR184 containing $\alpha 1$ (IV) collagen promoter and enhancer (Killen *et al.*, 1988). MCT cells (5×10^5) were rested for 24 h in serum-free medium and transfected with 10 μ g of plasmid DNA using DEAE-dextran (Segal, 1986). After 6–8 h, the medium was changed and mediators were added. All and agents modifying intracellular cAMP concentrations were added together in a single dose. For All-receptor blockade with saralasin, cells were pre-treated with saralasin for 6 h and subsequently incubated with All and a new dose of saralasin was added. After a total incubation for 24 hours, cells were harvested, washed in PBS, and lysed in 0.25 M tris(hydroxymethyl)amino-methane (Tris)-HCl (pH 8.0). The protein concentration in the supernatants was determined by the Lowry-method and equal amounts of protein were used for the chloramphenicol acetyltransferase assay (Rosenthal, 1987). [¹⁴C]chloramphenicol products were extracted in ethyl acetate, spotted on a silica thin layer chromatography plate and run in chloroform-methanol (95:5, vol:vol). Plates were autoradiographed and appropriate bands were cut out and counted for radioactivity ([¹⁴C]channel). Enzyme activities are calculated as relative CAT-activities with pA₀ = 1.

Statistical analysis

All data are presented as the means $\pm$ SD. Statistical significance between different groups were first tested with the nonparametric Kruskal-Wallis test for multiple comparison. If this test was significant, individual groups were compared using the Wilcoxon-Mann-Whitney U-test; $p < 0.05$ was considered as significant.

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